PCR amplification of phosphatonin includes DNA sequence coding for the first valine residue to the stop codon of phosphatonin (see Figure 8), plus linker sequence. A 5' overhang of the linker sequence is then generated by treating the PCR fragment with Pfu polymerase and dATP. Induction of fusion protein is carried by growing the cells and adding IPTG. The PCR conditions were as follows. Predenaturation; 95° C 3min, followed by 20 cycles of Denaturation; 95° C 45 sec, Annealing 59° C 60 sec, 72°C 2 min, and then 72° C 7 min final extension; followed by cooling 4° C. A Perkin Elmer 9600 thermocycler was programmed to carry out the PCR, and the following PCR buffer (PB), was used: 10mM Tris-HCl pH 8, 50mM KCl, 1 μM primers, 200 μM dNTP's. PB buffer was supplemented with 2 mM $MgCl_2$. For ligation independent cloning (LIC), the amplified product was then treated with pfu polymerase and dATP as described by Stratagene, and then directly annealed to linearized pCAL-n-EK plasmid vector with complementary linker overhangs. The construct was then transformed into competent E. coli XL1-blue mrf' cells, and competent E. coli BL21 (DE3) Clones were then selected on ampicillin plates, and plasmids prepared and sequenced. A summary of the vector and fusion construct is shown in Figure 14. High copy number plasmid is achieved with E. coli XL1-blue mrf' host, and high recombinant protein expression is obtained with E. coli BL21 (DE3).

4b. Purifying phosphatonin by calmodulin affinity resin

The method as described by Stratagene (cat~214405), can be used. Sequence upstream from the phosphatonin specific residues will contain a calmodulin binding sequence. Calmodulin resin is added to the crude cell lysate in the presence of calcium, and the protein allowed to bind. The slurry is then washed with calcium containing buffer, and the phosphatonin fusion protein eluted by addition of EGTA 2 mM in a Tris buffer (50 mM Tris-HCl pH 8). Removal of the calmodulin binding protein tag is then accomplished by digestion with site-specific protease EK, leaving pure recombinant human phosphatonin. Preferably, the method may be performed as follows (See table below for buffer compositions):

1. Cells are cultured and induced as described by the Stratagene protocol for pCAL-n-EK vectors (Cat No: #214405), using BL23 (DE3) E. coli host cells comprising plasmid p1BL21; see Figure 14.
2. Protein lysate is also prepared as described by the Stratagene protocol but using CCBB-II as resuspension buffer (resuspend cell pellet from 500 ml in 10 ml of CCBB-II). It is essential to sonicate in 30 sec pulses followed by 4 min cooling with ice. Tubes containing cells are kept on ice during sonication.
3. After sonication cells are spun at 10000g and the supernatant decanted. Most of the recombinant MEPE remains in the supernatant (protein-lysate).
4. The protein-lysate is then concentrated by using a VIVASCIENCE VIVASPIN (Cat No: VS1521 called 30,000 MWCO PES) concentrator with a 30000 molecular weight cut off. Approximately 8 ml of supernatant from 500 ml of cells concentrates down to 3.2 ml (X2.5 conc). Further concentration is not advisable.
5. For protein-lysate prepared from 190-200 ml of cells (~ 1.3 ml of equivalent protein-lysate), 1 ml of equilibrated calmodulin resin is then added (equilibrate resin as described by Stratagene using CCBB-II buffer).
6. The suspension is rotated overnight at 4° C.
7. The suspension is spun down (~ 3000 rpm on eppendorf centrifuge for 2 min), the supernatant removed and the resin resuspended in 1 ml of CCBB-II buffer.
8. The resin is spun down again and the first wash removed. This is repeated twice more (total of three washes in CCBB-II).
9. It is then washed once with WB-III; note non of the buffers including the final wash buffer contain detergents. The cells used for bio-assay are extremely sensitive to detergents even in trace amounts. WB-III is the same as CCBB-II but without protease inhibitors.
10. Non-specific proteins are eluted by washing with buffer EB-I twice (1 ml).
11. MEPE is eluted with EB-II 2-3 times (1 ml).
12. Protein is concentrated using a flowgen 10K microsep concentrator at 4° C. Generally 3 ml of MEPE eluate can be concentrated down to ~ 170 µl in 2 hr.
13. After running samples on an SDS-PAGE gel to assess purity and quantity multiple aliquots are made and frozen at -80°C. Repeated freeze thaw is avoided.

Buffers:

Component	CCBB-II	WBIII	EBI	EBII
Tris-Buffer pH 8	50 mM	50 mM	50 mM	50 mM
NaCl	300 mM	300 mM	150 mM	1 M
MgAcetate	1 mM	0	0	0
Imidazole	1 mM	0	0	0
CaCl2	2 mM	2 mM	0	0
Protease Inhibitors w/o EDTA	YES	No	No	No
EGTA	0	0	4 mM	4 mM

Protease inhibitor tablets were added 1 per 10 ml when used (Boehringer Mannheim), protease inhibitor w/o EDTA (Cat No: 1836 170). A final elution with 1M NaCl, EGTA (4 mM) buffer results in >95% purity of phosphatonin.

Example 5: Structure of phosphatonin

1. Primary structure and motifs:

The primary structure of the protein and the nucleic acid sequence are shown in Figure 8. The largest cDNA clone isolated for MEPE was 1655 bp and contained the entire 3' end of the gene with poly A^+ tail and a single polyadenylation sequence (AA[T/U]AAA) (figure 8). An open reading frame of 430 residues was found that overlapped and extended the other smaller MEPE cDNA clones isolated, with a predicted M_r 47.3 kDa and a pI of 7.4. The best fit consensus start codon Kozak, Nucleic Acids. Res. 15 (1987), 8125-8148), occurs at 255 bp, although two other methionines preceded this. It is possible that additional 5' sequence is missing, and an earlier start codon and or extended 5' untranslated sequence needs to be characterized. GCG- secondary structure prediction indicates that the protein is very hydrophilic with three localized areas of low hydrophobicity (figure 9). The protein has glycosylation motifs at residues 382 and 385 (NNST), and residues 383-386 (NSTR). There is also a glycosaminoglycan attachment site at residues 161-164 (SGDG). The approximate molecular weight without glycosylation is 54 kDa, and is in close agreement with the purified glycosylated form of (58-60 kDa). There are a number of phosphorylation site motifs (see Table 1), and these are predicted to play a role in the biological activity of the hormone or fragments thereof.